MICROBIOLOGICAL REVIEWS, Sept. 1987, p. 365–379
0146-0749/87/030365-15$02.00/0

Vol. 51, No. 3

Survival Strategies of Bacteria in the Natural Environment

D. B. ROSZAK† AND R. R. COLWELL*

Department of Microbiology, University of Maryland, College Park, Maryland 20742

INTRODUCTION

The need for methods to estimate accurately and reliably the total number of living bacteria in evironmental samples, including specific pathogens in aquatic systems, has not yet been met and therefore remains a continuing public health concern (20, 68, 146, 157). Since the turn of the century plate counts were, and in many situations remain, the method that is used to obtain a "total viable count," i.e., the total number of bacteria capable of multiplying at a given temperature, usually 35°C. The "total viable count" and other methods requiring growth of microorganisms present in samples being analyzed are most commonly employed to evaluate water quality and are usually performed in conjunction with analyses for coliforms. Standard microbiological methods are employed in measuring water quality, and the results are interpreted to indicate the extent of contamination by human and domestic wastes. The coliform bacteria, in general, remain the indicator organisms of choice to this day.

There is little illusion about the value of the coliform test. In fact, in the American Public Health Association manual (2) it is stated that methods provided in the manual are the best currently available; their limitations are clearly acknowledged. Modifications of the standard methods for measuring the microbiological quality of water and aquatic systems have been made over the years, including improvements in media and suggestions for alternative procedures, e.g., membrane filtration and spread plating to supplement the pour plating for bacterial counts. Progress has also been made in that enrichment and temperature acclimation have been proposed and employed in the multiple-tube fermentation test for coliforms (2).

In recent years, the heterotrophic plate count has come under increasing criticism because it is inefficient, at best, for enumerating viable bacteria present in marine and estuarine systems (Fig. 1). Large differences are consistently reported between results of plate counts and total direct microscopic counts, the latter usually accomplished with fluorochrome staining of physically intact bacteria (50). Also, incubation at 35°C of plates inoculated with samples of water collected from the aquatic environment is illogical, since temperatures of 35°C or greater rarely occur in temperate aquatic environments. Temperatures significantly greater than 20°C are often lethal for aquatic bacteria or for microorganisms adapted to the aquatic environment, including organisms of possible public health importance (118). Plate counts are also deficient in that no single medium will culture all bacteria in a sample, whether terrestrial or aquatic (153). Some investigators have taken the position that all methods of indirect enumeration, i.e., those requiring culture, are inherently selective and therefore deficient (29, 49).

Standard methods for aquatic and public health microbiology, in general, contain no provision for modification when saline water samples are to be tested. In fact, it is maintained that the same methods for freshwater are satisfactory for saline water (2), even though Olson (126), nearly 10 years ago, proposed a modification of the most-probable-number method designed to enhance detection of coliforms in marine and estuarine systems. Furthermore, it has been suggested that coliform "die-off" is overestimated because coliforms were "sub-lethally stressed and not detected in saline waters by the standard most probable number method." The plate count also has long been considered inadequate for applications in the terrestrial environment for many of the same reasons enunciated above (17, 25, 85, 146, 155, 157, 168, 176).

Not only is the efficiency of enumeration by standard methods held in question, but also their value in indicating public health safety is doubtful. Results of extensive epidemiological studies by Cabelli et al. (20) show a direct, linear relationship between swimming-associated, gastrointestinal illness and exposure to bathing water, even in waters in compliance with existing microbiological guidelines for recreational water (171). This finding suggests that currently employed standard methods are, indeed, inadequate to protect human health. Comparisons between results of plate counting, direct microscopic enumeration, and indirect activity measurements show that the number of bacteria capable of forming colonies on a solid medium is less than the number actually present and metabolically active, often by several orders of magnitude, in freshwater (17, 53), marine environments (70, 76, 100, 187), and soil (179) (Fig. 2). On the other hand, direct microscopic counts of samples collected from the natural environment and directly stained with fluorochromes do not provide reliable estimates of viability, i.e., the ability of the microorganisms viewed by this procedure to grow, metabolize, respire, or divide.

* Corresponding author.
† Present address: Molecular Biosystems, Incorp., 10030 Barnes Canyon Rd., San Diego, CA 92121.

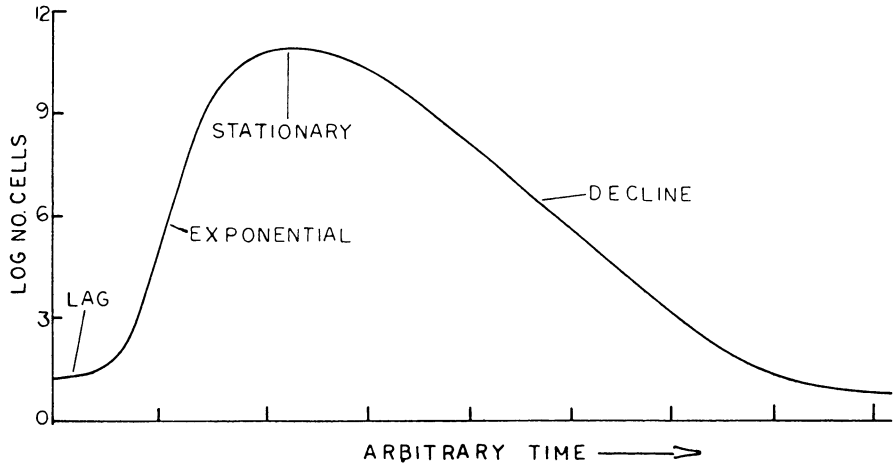

FIG. 1. Typical growth curve showing results of plate counting methods for estimating numbers of viable bacteria with respect to time.

Furthermore, indirect measurements of specific activities associated with viability, e.g., enzyme activity, photosynthesis, respiration, and energy charge, are too cumbersome and expensive for the routine sampling that must be done frequently in very large numbers. A direct relationship between the activity measured and number of potential pathogens present has not been found.

Recently, the existence of a viable but nonculturable stage, in which bacterial cells are intact and alive when tested by one or more of the above-mentioned criteria but do not undergo cell division in or on routinely employed bacteriological media, has been observed for many aquatic and marine bacteria. This phenomenon has recently attracted considerable attention, even though the problem of determining the metabolic state of those organisms observed in direct counts but not undergoing cell division was recognized as long ago as the late 1800s (80, 94). The phenomenon appears to reflect a mechanism of bacterial survival, with strain variation as well as variation in characteristics being related to environmental conditions and length of exposure to those conditions.

It is useful to review the observations reported during the past two decades that relate to the viable but nonculturable state. Jannasch (74) differentiated between those bacteria that merely survive and those that grow in marine waters. Nearly a decade later, Fliermans and Schmidt (46) combined autoradiography and immunofluorescence methods to identify metabolic activity of individual *Nitrobacter* cells that did not respond to laboratory culture. Dawes (33) interpreted endogenous metabolism as a survival strategy of microorganisms that endure for significant periods of time in the absence of nutrients. Hoppe (71) employed autoradiographic methods to demonstrate that free-living bacteria that did not form colonies on routinely employed bacteriological media were responsible, nevertheless, for the active metabolism of organic substrates.

Buck (17) questioned the use of the plate count, reviewed its various applications in ecological studies, and suggested alternatives for microbial enumeration. Concurrently, Kogure et al. (96) developed a direct viable counting method which permitted enumeration of substrate-responsive bacteria present in a sample. Cell division is inhibited by the

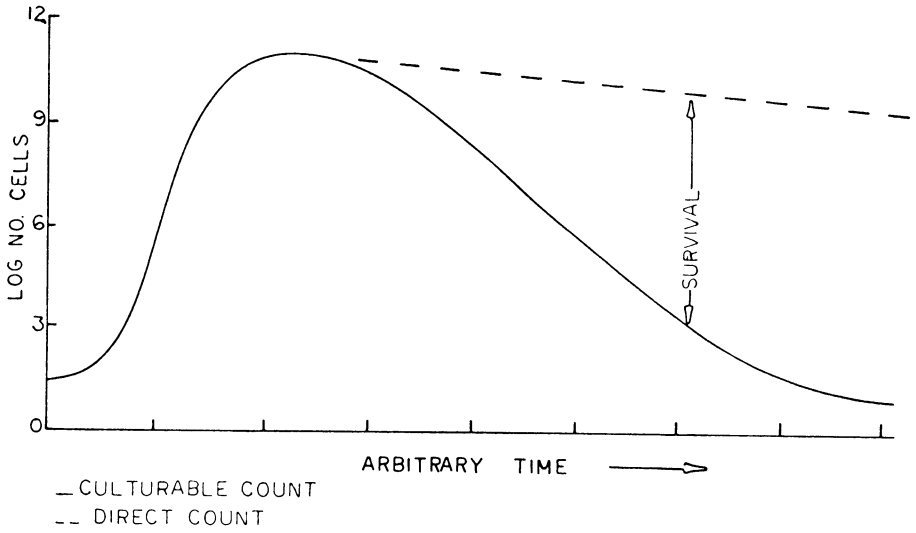

FIG. 2. Typical difference in number of bacteria estimated by direct viable counting with the standard plate counting method. The difference represents viable but not replicating cells.

addition of nalidixic acid while growth is enhanced by the addition of yeast extract; the result is large cells that can be easily differentiated from those that do not respond to the added substrate. Fry and Zia (53) demonstrated that up to 15% more viable cells of planktonic bacteria could be counted when slide culture methods were employed, compared with conventional plate counts. Xu et al. (185) stressed the need for reevaluation of the survival of pathogens in aquatic environments, taking into consideration the nonrecoverable stage of *Escherichia coli* and *Vibrio cholerae* in aquatic systems. They proposed that the concept of "die-off" of bacteria in the environment was not valid and that the concept of dormancy should be invoked since the cells, although unable to be enumerated by culture methods, remained viable.

Theories and definitions dealing with the subject of survival of bacteria in the environment versus "die-off" are controversial, because much of what has been learned recently about survival of bacteria during starvation in seawater (118) directly contradicts some of the conventional wisdom. This phenomenon recently was investigated by a quantitative approach employing new technology for detection of bacterial cells in the natural environment that has led to a more precise definition of the state.

THE "NORMAL" STATE OF BACTERIA

The microbial cell can be viewed as being in a dynamic state, adapting readily to shifts in environmental parameters by means of a wide variety of genotypic and phenotypic accomodations (62, 165, 166), such as appropriate modification of enzyme synthesis to take up growth-limiting nutrient, modulation of uptake rates for nutrients available in excess, rerouting of metabolic pathways to avoid possible blockages due to specific nutrient limitation, and coordination of synthetic rates to maintain balanced growth. These adaptive capabilities may, in fact, account for the ease with which microorganisms respond to culture conditions in the laboratory, which are often radically different from the natural habitat of the organism.

In the laboratory various aspects of microbial life are often partitioned and studied separately. Thus, a holistic view is frequently overlooked. For instance, microbiologists usually grow and maintain bacteria on standard laboratory media containing nutrients at concentrations that are very high, e.g., >2 g of C per liter, compared with the oligotrophic conditions commonly found in the natural environment, e.g., 1 to 15 mg of C per liter (110). Tests for the presence of oligotrophic bacteria employ such criteria as the ability to form colonies on a solid medium, to produce turbidity in a liquid medium, or to accumulate measurable levels of an end product or catalytic capacity to mediate a metabolic reaction. The results obtained from these growth tests give the appearance of precision and accuracy.

A set of reproducible results appears to describe a "normal" existence of bacteria. If a series of morphological and physiological changes occurs reproducibly in response to established conditions for growth, these changes were considered to comprise a "life cycle" of bacteria by some microbiologists (16, 181). In fact, the observed variations in bacterial morphology and physiology did occur, but the changes were concluded to be governed by imposed environmental factors rather than to inherently developmental (180).

As an example, a cell of a pathogenic bacterium in a nutrient-rich environment, namely, the human body, relative to its prior habitat in soil, water, or air, may respond by developing requirements for substrates or growth factors (or both) that in toto are relatively complex. A useful example to demonstrate this point is *Legionella* sp., which occurs in water but requires a complex medium and highly specific conditions for isolation and growth in the laboratory.

Not unexpectedly, the design of methods and media for use in medical laboratories does not take into consideration those conditions encountered by bacteria naturally occurring in or discharged into the natural environment, i.e., the in situ conditions of autochthonous bacteria. The clinical microbiologist often does not consider the conditions necessary to recover bacteria adapted to, or naturally occurring in, the environment (30).

Large populations of bacteria can be found in terrestrial and aquatic environments, and many of those recovered from soil and water are physiologically similar. However, nutrient concentrations and energy sources in terrestrial and aquatic environments usually are significantly different from those within the body of warm-blooded animals or, for that matter, in the gut of all animals. In truth, the wide range of conditions to which bacteria adapt precludes a single, "normal" set of conditions, evidenced by the difficulties faced in culturing autochthonous bacteria of extreme environments.

Heinmets et al. (65) observed that organisms considered to be nonviable because of failure to grow in a complete medium could, nonetheless, grow in the same medium after a short incubation period in the presence of suitable metabolites, e.g., 0.1% pyruvate, acetate, or oxalacetic acid. Heinmets et al. supported the theory that synthetic and metabolic growth processes continue in the absence of cell division under stressful conditions; they drew this conclusion from experimental evidence of filamentous growth of microorganisms in which the ability to divide was deliberately inhibited. The phenomenon was called "unbalanced growth" and was related to conditions in which DNA synthesis is temporarily blocked, preventing cell division, whereas other growth processes, including RNA and protein synthesis, continue. Cell mass increases under such conditions, resulting in lengthwise cell growth without division (40). Bretz (14), employing slide cultures to study survival of bacterial cells, reported that some cells, which he called moribund, were observed only to swell during the same period required for other cells to divide. The moribund cells eventually formed microcolonies and were therefore considered "viable."

Early studies in which bacteria were cultured under conditions simulating the oligotrophy typical of natural environments revealed that small, rounded bacteria developed under such conditions. These bacteria, which were smaller than those observed in laboratory culture media, were regarded as being "nutrient stressed." They developed to "normal" size upon provision of excess substrate (25, 31, 42, 70). Dawson et al. (36) labeled these "dwarfs" and concluded that they were forms of otherwise "normal" bacteria. MacDonell and Hood (103) cautioned that careful evaluation was needed before assuming this to be a "normal" stage of growth of bacteria, since in the natural marine environment many complex relationships exist among environmental parameters, namely, nutrient and salinity interactions and natural physiological responses to these parameters by autochthonous microorganisms.

The precise morphological and physiological condition of aquatic bacteria in the natural environment is clearly a controversial subject. Sieburth (152) suggested that the small, rounded forms found in aquatic systems are bacteria

in transition to the obligotrophic environment from a richer environment, such as agricultural runoff, sewage deposits, and fecal material, and they are in the process of starvation in the aquatic environment, being unable to sustain the earlier, i.e., "normal," rate of metabolism. Novitsky and Morita (125) claimed that a stress response or strategy occurs in native marine bacteria, comprising several "reductive" divisions, i.e., an increase in cell number without significant increase in biomass that decreases the size of the cells; the stress response, resulting in ultramicrobacteria, may be an important feature of truly autochthonous bacteria in the marine environment (118). Laboratory culture cannot be considered a "normal" environment for bacteria since conditions in natural environments do not always resemble standard laboratory culture media. Therefore, "starved" cells may more closely approximate a "normal" condition for bacteria than the overfed variety common under laboratory conditions (117).

NATURAL SYSTEMS

Most textbooks of microbiology present bacterial growth as a sequence of a lag period, an exponential phase, a stationary phase, and a decline or death phase (39). These phases are a condensation of seven periods observed in batch cultures and described as early as 1918 by Buchanan (16) as the (i) initial stationary, (ii) lag or growth acceleration, (iii) logarithmic growth, (iv) negative growth acceleration, (v) stationary, (vi) accelerated death, and (vii) logarithmic death phases. From studies of static cultures limited by various elements, e.g., phosphate, magnesium, carbon, or nitrogen, it was concluded that in general populations of bacteria in the death (or decline) phase reach an extended steady state, an eighth phase, with 2 to 4% of the cells remaining viable as measured by colony-forming ability (148). The latter stage is termed cryptic growth, the condition present when a portion of starved microbial populations dies, releasing products of lysis and leakage that support the growth of survivors (135). Dean and Hinshelwood (37) described this period as one of fluctuations in growth, with a periodic balancing of lytic and synthetic processes in the phase of decline. Investigations of bacterial growth were aimed primarily at the early phases (79, 181), whereas the negative growth or decline phase was often not addressed, for example, because the predominance of dead cells was of no interest in studies of growth (174) or because the negative growth phases presented distinct problems that required special methods to solve (114).

The so-called "death" phase of bacteria is of singular importance in microbial ecology. Aquatic and marine environments are subject to large spatial and temporal nutrient fluxes arising from seasonal and geographic variations in temperature, salinity, nutrient input, etc. Also, adjacent microhabitats may be very different even though in direct juxtaposition. Indeed, in the natural aquatic environment microbial habitats are abundant and varied, some consisting of expanses of liquid bounded by interfaces with solid, gaseous, or other liquid phases of different densities (91). The interfaces are sites where nutrients accumulate, allowing the microbes there to undergo active growth (36, 91, 108). Bacteria that are able to reach a nutrient-rich interface by chemotactic response, motility, or random movement have a selective advantage in an otherwise nutrient-poor environment. In addition to those bacteria able to attach to solid surfaces at a liquid-solid interface, other bacteria can loosely associate with surfaces temporarily by reversible sorbtion, thereby taking advantage of the high-nutrient environment adjacent to the actual surface (45, 109).

Small bacteria are usually the first colonizers of solid surfaces immersed in marine environments. They may arrive at the site in a metabolically dormant state, so a lag will occur before cell division (109). Shortly after, the small cells are replaced by actively growing bacteria that may be progeny of the same bacteria that arrived in a dormant stage (109). The sequence of events observed to take place in a dialysis chamber when a nutrient was provided at the solid surface to small starved cells within the chamber was first reversion to motility, followed by increase in cell size and only thereafter by division (91). The long lag before division after the addition of nutrient may reflect the time needed to achieve the proper size permitting exponential growth (91). Varied responses by different bacteria under conditions of starvation and addition of nutrient have been observed in oxygen uptake and size reduction (92), the latter serving to increase the surface/volume ratio (111). Kjelleberg et al. (91) hypothesized that marine bacteria are predominantly copiotrophic organisms requiring relatively high nutrient concentrations for growth and, therefore, exist as dormant cells adapted specifically to frequently and rapidly changing environments, thereby being able to take advantage of nutrients that tend to accumulate at interfaces. On the other hand, the native oligotrophs that are adapted to growth at low nutrient concentrations may not respond to high-nutrient zones.

To survive, bacteria in environmental waters must respond to a variety of physical and chemical variables, including low or high dissolved oxygen concentration, redox potential, and pH (154). These parameters frequently are less than optimal for bacterial growth and often the consequence is bacterial "die-off," i.e., the repeated finding that bacteria steadily decrease in numbers upon exposure to the natural environment when enumerated by culture methods over time and eventually are not recovered. It is the portion of the population that cannot be cultured, but can be observed by direct observation (the decline phase or "die-off" portion shown in Fig. 2), that we question in our studies of viable bacteria in aquatic systems. It can be debated whether these bacteria are transients in the ocean or autochthonous organisms temporarily not metabolizing (1, 70, 71, 125). Cohen and Barner (28) proposed that bacteria survive in media containing a paucity of specific nutrients, a condition that could be extrapolated to aquatic environments, where metabolites leach away and do not accumulate. Butkevich and Butkevich (18) determined that multiplication of marine bacteria depends on the composition of the medium and on temperature. They concluded that a considerable portion of the bacteria in the sea must be present in a resting stage.

Mandelstam (107) determined that nongrowing populations of *E. coli* were able to synthesize new enzymes at a rate approximately equal to protein breakdown, indicating that protein synthesis occurs at the expense of utilization of endogenous material. Kjeldgaard (90) reported that bacterial cells in the environment vary in size and chemical composition and concluded that the variation, in fact, represented the transition of cells from the resting state to the exponential growth phase, induced by fluctuations in environmental conditions.

Casida (24) described soil microorganisms possessing a cystlike capsule, observable by light diffraction microscopy, that had low endogenous activity but demonstrated the

ability to utilize exogenous substrates without delay at respiration rates lower than that in the vegetative cells.

Bae et al. (5), employing electron microscopy to investigate direct, uncultured soil samples, described dwarf cells, coccobacilli less than 0.3 μm in diameter, which comprised about 72% of the soil population obtained from five different locales in the New Jersey and Pennsylvania area. They concluded that these cells were different in size and fine structure from soil microorganisms grown in laboratory culture. In fact, laboratory culture usually upsets community balance by causing quick enrichment of certain portions of the population. Many of the cells in the natural samples were less than 0.08 μm in diameter and, therefore, were below the resolution of the light microscope. Only about 30% were found to possess cystlike or capsulelike structures. Nevertheless, none increased in size in the soil, but all remained intact.

Griffiths and Haight (58) suggested that high-affinity binding proteins were responsible for capture of amino acids in low-nutrient aquatic environments. The binding observed was at the level of picograms per liter, after the culture had been incubated for 72 days. This rate was even lower than observed in field studies in oligotrophic waters. The ability to scavenge substrates when they are available only in very low concentrations apparently provides a selective advantage to those organisms demonstrating this characteristic (115).

Because there are many different and often conflicting ideas about the state in which bacteria exist in natural waters, not to mention confusing and redundant terms used to describe these states, and problems with methods used to enumerate the bacteria, some of these ideas and terms are reviewed in the next section.

VIABILITY

The definition of viability of procaryotes is neither simple nor straightforward. Postgate (134) offered a pragmatic definition, namely, that viability is the property possessed by that portion of a bacterial population capable of multiplication when provided with optimal conditions for growth. However, what constitutes an "optimal environment" is not known for many native aquatic bacterial species and varies significantly with individual populations (134).

Jannasch (75) noted a lack of logic in enumerating and isolating bacteria present in seawater, since routine bacteriological methods require that microorganisms be transferred to media of relatively high nutrient concentration, on which visible colonies are produced within a short period of time. Plating on a relatively rich medium selects for those organisms that are probably atypical of low-nutrient habitats. In marine and freshwater environments, two basic types of populations of bacteria exist: those that can grow slowly at very low concentrations of nutrients, the oligotrophs, and the those that grow only at higher concentrations of nutrients but survive low-nutrient conditions by a variety of mechanisms, termed "eutrophs" or "copiotrophs" (110).

It was suggested by Jannasch and Jones (77) that, since many colonies became visible after 72 h of incubation, the use of more dilute media and longer incubation times, e.g., up to 7 days, would yield higher plate counts. Despite the best available information, there are many unknowns, e.g., how much time is necessary for incubation before pronouncement of death. It was generally believed that marine bacteria demonstrate variable generation times, e.g., between 10 min and 11 h, with a mean of about 4 to 5 h (182). Interestingly, in 1969, Jannasch (75) reported the isolation of aquatic bacteria that had a mean generation time of 53 h, and he estimated the in situ range for generation time to be from 20 to 200 h. Carlucci and Williams (23) calculated a doubling time of 210 h for organisms isolated from the marine environment at a depth of 5,500 m and an in situ temperature of 5°C. Compare this, then, to the estimated in situ generation time for *E. coli* of 12 h in the intestine, which can be considered a eutrophic habitat.

Certain factors, including the following, have been associated with spurious or erroneous viable bacterial counts. Sublethally stressed populations, i.e., bacteria that are slightly impaired or damaged by environmental conditions but that are not dead (119), are believed to be more susceptible, in general, to changes in the physicochemical microenvironment and therefore may exhibit greater variability in response to added nutrient (47). Furthermore, selective media, traditionally employed in standard culturing procedures, are not optimal for growth even for nonstressed populations, because inhibitory substances such as dyes comprise the selective design of those media. The use of solutions of metabolites to restore the viability of artifically inactivated bacterial cultures was reported in 1953 (65). More recently, methods employed to resuscitate "stressed" bacterial cells have received significant attention, with dilute, nonselective media and incubation temperatures allowing acclimation to the test conditions being used (47, 61, 72, 143–145). The majority of studies of "resuscitation" have been directed toward increasing the number of cells that can be cultured from natural samples, with the ultimate objective being to approximate more closely the number of cells observed with direct staining methods.

Valentine and Bradfield (173) proposed the term "viable" to describe cells capable of multiplying and forming colonies, but suggested the term "live" for cells showing other signs of viability, such as respiration, even if the cells were unable to divide under the prevailing conditions. This might lead one to consider that viable cells are not alive, which is, obviously, not so. Later experiments utilizing radiolabeled substrates to measure heterotrophic uptake and respiration confirmed the existence of such living populations of cells (46, 84, 112). Kurath and Morita (100) referred to cells as being viable only so long as they demonstrated the capacity to reproduce on an agar medium suitable for growth of the organism. They were described as "nonviable" once the cells lost the ability to form a colony. While applying this definition, Kurath and Morita (100) nevertheless concluded that the subpopulation of cells that were actively respiring existed at a concentration 10-fold greater than the number of "viable" cells (by their definition). Similarly, Hoppe (70, 71), who employed autoradiographic methods, observed that actively metabolizing bacteria that did not form colonies on routine agar media represented the predominant inhabitants of offshore marine regions. Since such heterotrophic microbial populations cannot be described completely by direct or colony counts, Hoppe (70, 71) concluded that bacteriological methods based on the total number of actively metabolizing bacteria should be used.

With slide culture as the detection method, culture is still required, but it provides a means for monitoring cell division by microscopic observation. Postgate and Hunter (137) described dead bacteria as those that did not divide conceding however that the other, nondividing bacteria may be in some sense "alive," because they retained their osmotic barriers after "death." Death in this case was defined as the lack of cell multiplication.

Most of the experiments carried out to quantify bacteria have been aimed at measuring viability of bacterial populations. Often these studies were done after application of "stress" or induction of "injury," meaning the imposition of conditions including nonpermissive heat, cold, drying, pressure, salinity, chemicals, or radiation. Much of this research has been reviewed recently by Andrew and Russell (4).

As indicated by Postgate (133), microorganisms are not analagous to multicellular organisms in undergoing a natural senescence and death sequence. In contrast to cells of higher organisms, which are known to senesce and die over a limited number of doublings, most bacteria can continue, in principle, to divide indefinitely. The condition closest to senescence in bacteria might be reached when the bacteria are exposed to starvation and achieve a transient state between viability and death when they are functioning but incapable of multiplication, a state Postgate (133) termed "moribund." The same term was applied to bacterial cells studied by Bretz (14), who used slide culture methods to differentiate between living and dead populations. The moribund cells were observed only to swell during the same time interval during which other viable cells were able to divide, but eventually these moribund cells did produce microcolonies and were considered viable.

The conventional view of many microbiologists is that a single, viable microorganism is represented by a cell capable of dividing and forming at least one live daughter cell when it is placed in a favorable environment. Dormancy has been defined rather tidily as a "rest period" or "reversible interruption of the phenotypic development of an organism" (160). A dormant stage has been documented and readily accepted by most investigators as being applicable to bacteria that form spores or cysts but not necessarily for nonsporulating bacteria. Many bacteria found in the aquatic environment are able to persist for significant periods of time as vegetative cells, exhausting energy reserves at a very slow rate via lowered metabolic activity (124). A dormant stage was proposed to account for the fact that some organisms are not transient in soil and water but demonstrate seasonal distribution patterns also for animal and plant pathogens which promptly "disappear" or "die back" upon entry into natural waters or soil (57).

A study of *Klebsiella aerogenes* maintained under starvation conditions, i.e., in nonnutrient buffer (21), demonstrated that after 24 h ca. 20% of the population was viable by culture methods. However, the remaining 80% of the population was intact and responsive to mild changes in medium composition and concentration, suggesting the cells were viable but nonculturable. These results led to the formulation of the concept of a "pseudosenescent" state, in which bacteria lose the ability to multiply as a result of certain stresses but remain completely functional as individuals (135). Earlier, Jannasch (74) suggested that, below certain threshold concentrations of substrate, two strategies for survival were observed: (i) the ability to grow at low substrate concentrations and (ii) the ability to become temporarily inactive (nonculturable) but to survive. In chemostat cultures, the second strategy resulted in "washout" of the population. Since the cells were not multiplying below the threshold amount of nutrients, the steady state of the chemostat could not be maintained and the flow of the chemostat steadily removed the bacterial population, based on culturability criteria. Taking all evidence into account, it is concluded that living, metabolically active, bacterial populations exist that do not form colonies on agar plates in the laboratory and therefore exhibit no "viable count."

Interest in the problem of enumeration of viable organisms that do not grow on standard media has induced research on enumeration methods, such as radiolabeled substrate uptake, methods for detecting respiring cells, and differential staining procedures. Clearly, the viable but nonculturable bacterial populations cannot be enumerated by currently employed culturing methods, because the populations do not grow on conventional culture media, but their quantitation is possible by methods that do not require culturing.

SURVIVAL

Survival has been defined as maintenance of viability under adverse circumstances. Less clear is what constitutes "viability" and what constitutes "adverse." Among microbial survival mechanisms is the formation of spores and cysts by soil microorganisms (158). There are also microorganisms that neither sporulate nor encyst yet can be isolated from unfavorable environments. Such microorganisms persist as vegetative cells but use up their energy reserves slowly as a result of lowered metabolic activity (125).

Stevenson (158), in what is now considered to be a classic discussion of dormancy, expressed the belief that there exist certain stresses, such as starvation, that lead bacteria to decrease in size and activity and that once a particular stress has been removed normal development, prior to the stress, ensues. According to Jannasch (76), microbial multiplication in natural habitats must be assumed to alternate with dormancy. This view does not take into consideration shifts in metabolism in response to changing nutrient levels, but proposes just two states. The common observation in low-nutrient habitats of large direct microscopic bacterial numbers but corresponding low bacterial numbers measured by culture methods (19, 77) suggests that large portions of microbial populations are indeed dormant or undergoing a shift in metabolism in response to changing environmental conditions. Interestingly, Vaccaro and Jannasch (172), in 1966, demonstrated that natural populations of microorganisms in seawater tended to respond kinetically to organic enrichment within a period of about 12 h.

The ability to reduce the endogenous metabolic rate rapidly may be a primary survival requirement if bacteria are to endure during starvation conditions. Many investigators (11, 33, 100, 122, 125, 141) have suggested that reduced respiration and low endogenous metabolism are mechanisms enhancing survival under sparse nutrient conditions. Using starvation-resistant isolates, i.e., strains selected for longevity by culturing in growth chambers with phosphate buffer, Jones and Rhodes-Roberts (84) found that various physiological properties, notably, nitrogen-scavenging ability (83), formation of intracellular polyhydroxybutyrate or protein reserves (33, 35, 147), possession of abnormally low cellular protein content, low maintenance energy requirement (131, 167), or state of low energy charge (26, 115), could not account for the observed resistance to starvation. Jones and Rhodes-Roberts (84) proposed that adaptation to the normally low nutrient aquatic environment is an innate characteristic of marine bacteria.

In fact, the ability to function at a very low metabolic rate is not unique to marine bacteria, but has been observed to occur in soil bacteria and is considered to be an adaptive mechanism whereby soil bacteria survive conditions of extreme nutrient limitation (12, 64, 116).

Resting forms of physiologically and genetically diverse groups of bacteria such as species of the genera *Myxococcus* (121), *Azotobacter* (147), *Streptomyces* (156), *Thermoactino-*

myces (52), *Bacillus* (7), and *Clostridium* develop under conditions of nutrient limitation. Limiting carbon, nitrogen, or phosphorus leads to spore development, which is not a reproductive state but a survival state. The spore state is very resistant to destructive agents such as temperature, desiccation, pesticides, antibiotics, and dyes (89). The water content of the spore is very low and metabolism is reduced to nil while cells remain viable, as evidenced by germination upon return of favorable conditions. The dormant spores have a low energy charge and contain a low concentration of nucleoside triphosphates as well as pyrimidine nucleotides and acetyl coenzyme A (151). Phosphoglycerate and basic proteins seem to be the primary energy source of ATP synthesis during the first few minutes of germination, when ATP is rapidly synthesized before macromolecular synthesis is seen. Germination of spores varies, depending on the conditions that initiated sporulation and conditions during the spore stage. However, agents implicated in initiating germination are nutritional, enzymatic, chemical, and physical: specifically, sugars, amino acids, purines, salts, detergents, calcium, dipicolinic acid, and lysozyme (56, 90). An interesting stage in germination is activation of the spore. Freshly formed spores require some stress, such as radiation, low pH, heat, or reducing agents, for initiation to take place, even if optimum conditions are provided; however, aged spores will germinate without activation. Once activated, the dormant cell can generate ATP, synthesize RNA, and assemble amino acids into proteins initially at the expense of stored compounds. Sporulation is a transition between the vegetative cell and the spore and does not occur abruptly but as a shift in the balance of events that occur in response to the nutritional deprivation (48). Commitment to the spore stage occurs at some point but seems to be a progression of events which, once completed, prevents reversion; the point of no return is the accumulation of successive survival events. These events, which are well established for spore-forming bacteria, may be analogous to the dormant stage seen in viable but nonculturable cells, with the only real difference being the lack of an obviously different morphological condition, i.e., spore structure.

The suggestion that changes that bacteria undergo in response to starvation conditions are associated with depletion of cellular reserves, with less essential material being scavenged for essential needs, was made by Tabor et al. (163) in examining cryptic growth, during which survivors in a "dying" population survive by utilizing the leakage and lysis products of dead organisms (137). Indeed, autochthonous aquatic bacteria might be expected to possess such capabilities, even though some allochthonous bacteria may also employ similar strategies for survival under conditions encountered in an unfavorable environment (149).

It has been demonstrated convincingly that some bacterial species that are autochthonous to the marine environment may undergo what has been described as "reductive division," in response to exposure to carbon and nitrogen starvation, thereby yielding a population of daughter cells proportionally reduced in size with an increased surface/volume ratio (123). Modulation of endogenous respiration, the result of which is a lowered rate of metabolism and initiation of macromolecular degradation, provides efficient utilization of limited endogenous substrates (13, 124).

Under starvation conditions, i.e., maintenance in unsupplemented phosphate buffer, the energy of maintenance, which is defined as the energy consumed for purposes other than production of new biomass, such as osmotic regulation, maintenance of intracellular pH, turnover of macromolecules, and motility, must be supplied by endogenous substrates (12). Therefore, a low rate of endogenous metabolism can serve to prolong the viability of an organism under adverse conditions (33). Bacteria that have the greatest capacity for longevity have been shown to be small, in comparison to laboratory-grown strains, and to possess correspondingly lower metabolic rates (64). As pointed out by Dawes and Ribbons (34), the characteristic low metabolic rate is shared with spores, which exhibit a capacity for survival and a barely detectable metabolic rate. It has been hypothesized that these responses represent survival strategies for bacteria in the marine environment and that the ability to exhibit such responses provides the bacteria with a selective advantage (125).

Carlucci (22) reasoned that since organic solutes can be shown to be present in the open ocean in concentrations of only microgram quantities of carbon per liter, small, rounded cells may reflect a "normal" morphological and physiological state in this environment. Sparsely available energy sources in the sea present bacteria with a feast-or-famine situation and little opportunity for feast conditions, as in the oligotrophic environments discussed by Poindexter (132). Using strain ANT-300 as an example of a marine psychrophile, Carlucci (22) suggested that certain species appear to possess genetic information necessary to endure protracted periods of extreme nutrient scarcity, typical of that encountered by bacteria in the oceans. However, no experimental evidence was presented to support this theory.

Guelin et al. (60) observed the "rounding up" phenomenon as well as a concomitant decrease in cell volume at initial exposure to organic compound-free conditions. The "round body" phenomenon in *Vibrio* spp. was described by Felter et al. and Kennedy et al. (43, 44, 88) and reported by Baker et al. (6) for starving *Vibrio cholerae* cells. In these studies small cells were observed to adjust to substrate-limited environments by appropriate physiological responses and thereby to endure stressful conditions imposed by the natural environment.

In the early 1950s, Bisset (9) claimed that nearly all bacteria possess resting or specialized distributive stages which he termed "microcysts." These microcysts were considered to be a survival stage in the life cycle of certain *Vibrio* spp., serving to perpetuate the species through periods of extreme adversity (61).

The morphological changes that occur within the cell when some cells "round up" (6, 36, 60, 88) and decrease in volume from 15-fold (60) to 300-fold (113) have been reported to be typical physiological responses of some bacteria upon exposure to organic nutrient-free conditions. Stevenson (158), in reviewing the occurrence of dormancy in bacterial cells, maintained that the small ovoid forms of bacteria, normally observed in natural systems by direct microscopy, represent exogenously dormant forms in delayed development, responding to unfavorable chemical or physical conditions in the environment. Although this is an acceptable explanation, it can be expanded, and we suggest that this is a sporelike or "somnicell" stage for non-spore-forming bacteria.

It is reasonable to postulate that there is a continuous response of cells to the environment, rather than specific range-limited stages. The open ocean environment is continuously oligotrophic, with only brief periods of nutrient input; thus, bacterial cells can be expected to respond linearly with time spent in such an environment. Whereas cells with a history of recent exposure to a given environment might have a more conservative response to low-nutrient condi-

tions, those with prolonged exposure to the same conditions would be expected to employ more extreme strategies for survival.

There are many mechanisms that promote survival in changing natural systems, yet fluctuations in the number of bacteria in natural aquatic systems related to seasonal and geographical distribution, as assayed by plate culture methods, reveal consistent patterns that correlate with selected physical and chemical parameters (101). These patterns suggest that bacteria exist in a nonculturable form at certain times of the year but remain viable, and that when appropriate conditions occur, the bacteria return to a culturable state.

ULTRAMICROBACTERIA

Cellovibrio spp. occur in the natural environment as small filterable particles that cannot be subcultured or reverted to normal cells (170). *Aerobacter* spp. have also been found to form starvation-resistant, small cells (64). Dwarf cells, some less than 0.08 μm in diameter, have been observed in soil samples with electron microscopy (5). In the last case, many of the cells were too small to be observed by light microscopy and possessed fine structural differences from normal-sized soil bacteria. Anderson and Heffernan (3), using a double-filtration method, isolated bacteria from seawater, the size of these bacteria was such that they passed through a 0.45-μm filter and were retained on a 0.22-μm filter. These were subsequently identified as *Spirillum*, *Leucothrix*, *Flavobacterium*, *Cytophaga*, and *Vibrio* spp.

The first definitive report, with laboratory culture, of the production of small cells by starvation was of the response of the marine psychrophilic bacterium, strain ANT-300, to conditions of prolonged nutrient limitation (123–125). The study showed that a population of initially low cell density increased in cell numbers and that, after 70 weeks under nutrient limitation conditions, 15 times the original number of cells were found to be viable. The conclusion was that ANT-300 was particularly suited to survival at low nutrient concentrations and low population densities by sustaining an increase in cell number. In 1981, Torrella and Morita (169) characterized a form of bacteria that they named "ultramicrobacteria." These were defined as small in size, i.e., less than 0.3 μm in diameter, demonstrated slow growth, and did not significantly increase in size when inoculated onto a nutrient-rich agar medium. For many of these isolates, biochemical characterization was not performed due to lack of sufficient growth of the organisms.

The need for minimal nutrient concentration and a prolonged incubation time appears to be extremely important for recovering ultramicrobacteria (163). Torrella and Morita (169) also expressed the view that the laboratory concentrations of nutrient employed in their study were inappropriately rich, because it was hypothesized, isolates were conditioned to extreme nutrient limitation. MacDonell and Hood (103, 104) recovered minute spherical bacteria that appeared to require conditioning to higher nutrient concentration before the higher concentration of organic matter could be utilized as a substrate for growth. The response elicited by the organisms to increased nutrient concentration was an increase in cell volume and in growth rate, with eventual formation of visible colonies. The failure of ultramicrobacteria or other native environmental isolates to grow immediately on rich media may be related to substrate-accelerated death, as speculated by Postgate and Hunter (137). Under laboratory conditions, a population growing under limited substrate conditions exhibits rapid cessation of growth when presented with that substrate under starvation conditions. However, the phenomenon of substrate-accelerated death has usually been demonstrated by using relatively large concentrations of a single nutrient, such a glucose or ribose (21, 138, 167). It is unfortunate that direct microscopy was not employed to determine the total number of cells, because that would have provided a clue to the presence of viable but nonculturable cells.

From these studies, we conclude that the ultramicrobacteria are representative of the autochthonous bacterial communities in the marine and estuarine environment. The ultramicrobacteria are, undoubtedly, a portion of the bacteria that are not recovered by standard culture methods and may represent an early strategy in a linear progression of survival mechanisms.

DETECTION

Many methods have been employed to isolate and study cells that are viable but unable to multiply (15, 67, 161, 178, 183). Radiolabeling techniques have been employed, both alone and in combination with other methods such as fluorescent labeling, to estimate cellular metabolic activity (46, 84, 112, 130, 161). In all studies, cells are exposed to an appropriate, radioactively labeled substrate. Only cells that are metabolically active and able to incorporate the radioactive substrate are detected on photographic film or enumerated with a scintillation counter.

Autoradiography frequently has been combined with other methods such as immunofluorescence and has been found to be useful for distinguishing specific cells. By microscopic examination, stained cells and silver grains in a film emulsion can be viewed in the same microscopic field by adjusting the light and plane of observation (46, 112, 161).

The selectivity of culture methods is established; other methods proposed for enumerating viable microbial populations are numerous (Table 1) and are, in general, based on the assumption that there is a difference between living and dead cells. Some methods have been developed with the assumption that differences can be observed by staining, including use of methylene blue, toluidine blue (175), neutral red (94), and fluorescent dyes (27, 70, 78, 81, 159). Although many of the methods work well when used with artificially mixed populations of living and killed cells, "intermediate" forms such as those observed in natural aquatic populations yield ambiguous results, which is an important finding relative to the viable but nonculturable state discussed here (see above).

One method, the acridine orange direct count (32, 50, 69), has been widely adopted and is generally accepted as being reliable for direct enumeration of total bacterial populations (10, 82). From results obtained with the acridine orange direct counting method, it has been suggested that it could be possible to distinguish between living (red fluorescence) and inactive (green fluorescence) cells (99, 159), based on the knowledge that acridine orange intercalates with nucleic acids and fluoresces red-orange in association with RNA and green in association with DNA (69). A high RNA/DNA ratio indicates active metabolism, whereas a low ratio indicates metabolic inactivity. Correlating red fluorescence with active cells and green fluorescence with inactive cells may be valid; however, factors other than the ratio of nucleic acids, such as pH of dye preparation, incubation time with acridine orange, and concentration of the acridine orange, have been shown to influence the fluorescent color. It is therefore not

TABLE 1. Methods described for differentiating living from dead bacterial cells

Method	Protocol	Source, year, and reference
Differential staining for microscopic observation	"Old" vesuvin solution added to leukocyte exudate containing bacteria (live cells, no change; dead cells stain brown)	Metchnikoff, 1887 (94)
	Neutral red stain added to bacterial solutions (live cells, no change; dead cells stain red)	Erlich, 1894 (94)
	Eosin-methylene blue stain added to wet mount (cyanophilic live cells stain blue; eosinophlic dead cells stain red)	Bordet, 1895 (94)
	Bacterial suspension added to neutral red and methylene blue film on microscope slide (live cells stain violet red; dead cells stain blue)	Ruzicka, 1905 (94)
	Dried and fixed bacteria smear stained with carbol fuchsin and methylene blue solution (live cells stain blue; dead cells stain red)	Proca, 1909 (94)
	As above, but with spore suspensions (live spores do not stain, dead spores stain blue)	Proca and Danila, 1909 (94)
	Dried and fixed bacterial smear stained 5 min with methylene blue, rinsed, stained 5 to 10 s with 1:10 carbol-fuschin (live cells stain blue; dead cells stain red)	Kayser, 1912 (94)
	Methylene blue solution applied to yeast cell preparation (no change in reproducing cells; nonreproducing cells stain blue)	Fraser, 1920 (51)
	Neutral red added to bacterial suspension (live cells remain colorless; dead cells stain red)	Seiffert, 1922 (94)
	Congo red applied as negative stain to bacterial suspensions (live cells remain colorless; dead cells stain red)	Henrici, 1923 (66)
	Congo red and methylene blue added to fermenting cultures (live cells remain unchanged; nonreproducing, nonfermenting cells take up stain)	Rahn and Barnes, 1933 (14)
	Gram stain applied to gram-positive cells (live cells stain blue; dead cells stain red)	Frazier and Boyer, 1934 (94)
	Hanging blocks of agar inoculated with bacterial suspension and observed microscopically for multiplication for four generations	Kelly and Rahn, 1932 (87)
	Individual cell multiplication on solid medium followed by photocinematography	Bayne-Jones and Adolph, 1932 (87)
	Growing microorganisms examined microscopically by oblique incident illumination in a moist chamber on solid nutrient surface	Pearce and Powell, 1951 (128)
	Culture chamber containing cellophane membrane inoculated with individual microorganisms observed for multiplication	Harris and Powell, 1951 (63)
	Graticulated cellophane membrane inoculated with bacterial culture; microscopic counting of organisms before and after a short incubation period	Powell, 1956 (139)
	For orientation, Formvar grid replicas inoculated with bacteria and periodically observed microscopically for multiplication	Taubeneck, 1958 (164)
	Microcolony formation on membrane filters of small cells observed microscopically	Jannasch, 1958 (73)
	Organisms dispersed in thin agar films suspended in wire loops immersed in liquid medium; after various incubation periods films are stained and observed microscopically on slides for number of bacteria per colony	Jebb and Tomlinson, 1960 (79)
	Short-term incubation of bacteria on agar films followed by differentation by microscopic counting of number of microcolonies per number of total bacteria	Postgate et al., 1961 (136)
	Agar films on blotting paper in chambers inoculated with bacterial suspensions and incubated 1 to 6 h; phase-contrast microscopic observation of growth of viable and moribund cells	Bretz, 1962 (14)
	Cellophane membrane inoculated with bacteria in a continuous flow through chamber containing liquid synthetic medium observed microscopically for seven generations	Quesnell, 1963 (140)

(Continued on next page)

TABLE 1—*Continued*

Method	Protocol	Source, year, and reference
	Microculture, following clones of antibiotic-treated cells for inhibition of multiplication	Kogut et al., 1965 (98)
	Agar blocks containing bacterial suspensions observed by time-lapse, phase-contrast photomicrography for microcolony formation	Torrella and Morita, 1981 (169)
	Agar films contained in metal rings on microscope slides inoculated with concentrated freshwater sample, incubated, and observed microscopically for microcolonies and single cells	Fry and Zia, 1982 (53)
Refactive index	Gelatin solutions abolish light scattering by dead cells, leaving live cells visible	Barer et al., 1953 (8)
Optical effect	Optical density of a suspension of live bacteria is greater in a saline solution than in distilled water; dead bacteria show no change	Mager et al., 1956 (105)
	Optical density is 25% greater for live organisms in saline than in distilled water, whereas killed organisms show no effect; mixtures show optical effect in proportion to % live organisms from +25% to −10% of optical density in water; used as a measure of intact osmotic barrier required for living organisms	Postgate and Hunter, 1962 (137)
Constituent leakage	Dead bacteria in culture leak nucleotides; measure labeled nucleotides in culture to estimate amount of dead cells	Koch, 1959 (95)
Division inhibition	Medium containing 3% urea promoted growth but inhibits division of bacteria; electron microscopic observation differentiates growing from dead bacteria	Valentine and Bradfield 1954 (173)
	6-h incubation of bacteria in yeast extract and nalidixic acid promotes growth while inhibiting cell division in gram-negative bacteria; microscopic counts after incubation of acridine orange-stained preparations differentiate elongated growing cells from short nongrowing cells	Kogure et al., 1979 (96)
	As above, but various Casamino Acids, tryptone, glutamate, and glucose as growth substrates	Peele and Colwell, 1981 (129)
	Kogure method with staining by fluorescent antibody for specific population enumeration	Roszak et al., 1984 (149)
Respiration	Microscopic observation of bacteria treated with triphenyltetrazolium for reduced products indicative of dehydrogenase activity	Weibull, 1953 (177)
	Electron microscopic observation of bacteria treated with tetranitroblue tetrazolium for reduction products indicative of dehydrogenase activity as criterion for viable cell enumeration	Sedar and Burde, 1965 (150)
	Reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan results in an accumulation of dark red spots which attain size and degree of optical density to allow observation by light microscopy (respiring bacteria contain spots; nonrespiring cells do not)	Zimmerman et al., 1978 (186)
	As above, but counterstained with malachite green for additional contrast	Dutton et al., 1983 (41)
Microautoradiography	[^{3}H]glucose, [^{3}H]acetate; incubation of bacterial sample with radiolabeled compound and eventual filtration of samples, exposure of filter to photographic film, development of film, microscopic viewing of film for decay products of radioactive compounds identified by darkened grains on film	Munro and Brock, 1968 (120)

(Continued)

considered valid to employ color of fluorescent staining to distinguish between living and dead cells (69).

Actively metabolizing, respiring bacteria can be assayed and enumerated by their ability to reduce 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride to 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride–formazan, which is deposited as optically dense, dark red, intracellular aggregates that become large and dense enough for observation by light microscopy. This reaction can be used to estimate the viability of cultures (186). Although it is

TABLE 1—*Continued*

Method	Protocol	Source, year, and reference
	[^{3}H]glucose, [^{3}H]thymidine; film emulsion on slides, cells stained with methylene blue	Ramsey, 1974 (142)
	Scanning electronic microscopic observation of bacteria on detritus after incubation with radiolabeled [^{3}H]glucose and [^{3}H]acetate	Paerl, 1974 (127)
	$^{32}PO_4$; radiographic films microscopically observed and counted for number of spots per sample volume	Peroni and Laverello, 1975 (130)
	$NaH^{14}CO_2$ and immunofluorescence staining by fluorescent antibody combined to identify organisms containing radioactivity	Fliermans and Schmidt, 1975 (146)
	^{3}H-amino acid mixture, X-ray film; count spots per ml of sample	Hoppe, 1976 (70)
	[^{3}H]acetate, [^{3}H]glucose, [^{3}H]leucine; stripping film; filter stained and viewed separately for placement of cells	Hoppe, 1978 (71)
	[^{3}H]glucose; film emulsion on slides; acridine orange staining of cells	Meyer-Reil, 1978 (112)
	[^{3}H]acetic acid, ^{3}H-amino acid, [^{3}H]thymidine; acridine orange staining of cells on film emulsion, removal of filter, observation of cells and developed grains in same plane of focus	Tabor and Neihof, 1984 (162)

applicable to many bacterial populations, if one is enumerating cells in environmental samples, the cells are often very small and difficult to distinquish from inanimate objects, making the problem of detecting intracellular deposits unmanageable. A modification of this method involves the addition of malachite green as a counterstain to improve the contrast between cell aggregates and the background (41). However, only marginal improvement is achieved for what must be concluded to be inadequate methodology for enumeration of respiring bacteria in environmental samples.

Although it is agreed that plate counts are unsatisfactory for estimation of the total number of viable bacteria present in environmental samples (17, 68, 77, 80), microcolony methods employing slide culture appear to be somewhat more effective. However, there is a practical limitation in that slide culture preparation is a tedious procedure, and the prolonged observation that is required remains an impediment, especially if large numbers of samples must be screened (57, 136). The possibility of automating the procedure could eliminate this drawback. Additional problems, however, are the same as those encountered in conventional plate counts, such as limitation in the selection of culture medium and uncertainty of the appropriate length of incubation time. An advantage of slide culture over conventional plate counts is that the criterion for viability is replication of a single cell and not formation of a macrocolony, which can arise from the division of cells in a clump.

An unique and useful method was developed by Kogure et al. (96, 97) to overcome the problem of underestimation of viable cells by plate counts and overestimation by direct microscopy. To estimate directly the viable bacteria present in seawater Kogure et al. preincubated samples with small quantities of nalidixic acid and yeast extract. Nalidixic acid, a specific inhibitor of DNA synthesis, prevents cell division in gram-negative bacteria by inhibiting DNA replication and thus preventing cross-wall formation (38, 54, 55, 86, 102). Other synthetic pathways, however, continue to function. The result is formation of elongated, metabolically active cells in the presence of nutrient. This direct viable count allows enumeration of cells which are actively growing as well as dormant cells which are physiologically responsive. Peele and Colwell (129) experimented with a variety of substrates and obtained results indicating that growth requirements and specific substrate preferences of marine bacteria can affect direct microscopic counts of substrate-responsive cells. The method has been compared with other viable counting methods (106) and has been employed in environmental studies (59, 129, 149, 185). Combined with immune fluorescence staining, it can provide a useful detection procedure for specific bacterial species in the environment (184).

It should be noted that nalidixic acid is effective only against susceptible gram-negative bacteria; some gram-negative bacteria are resistant to the antibiotic, potentially causing underestimation of viable populations. Antibiotics with similar function or combinations of antibiotics are currently being investigated and may expand the usefulness of this method.

ACKNOWLEDGMENTS

We gratefully acknowledge R. Y. Morita, K. Kogure, J. Poindexter, and A. Pearson for helpful discussions.

The research reported in this review was supported in part by grants from the Office of Naval Research (ONR N000-14-81-K0638), the Environmental Protection Agency (EPA cooperative agreement CR81346-01-0), National Science Foundation (NSFBSR-84-08418), and University of Maryland Sea Grant College (NA84-AA-D-0014).

LITERATURE CITED

1. **Akagi, Y., U. Simidu, and N. Taga.** 1980. Growth responses of oligotrophic and heterotrophic marine bacteria in various substrate concentrations and taxonomic studies on them. Can. J. Microbiol. **26**:800–806.
2. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Inc., Washington, D.C.
3. **Anderson, J. I., and W. P. Heffernan.** 1965. Isolation and characterization of filterable marine bacteria. J. Bacteriol. **90**:1713–1718.
4. **Andrew, M. H. E., and A. D. Russell (ed.).** 1984. The revival of injured microbes. Academic Press, Inc., London.

5. **Bae, H. C., E. H. Costa-Robles, and L. E. Casida.** 1972. Microflora of soil as viewed by transmission electron microscopy. Appl. Microbiol. **23**:637–648.
6. **Baker, R. M., F. L. Singleton, and M. A. Hood.** 1983. The effects of nutrient deprivation on *Vibrio cholerae*. Appl. Environ. Microbiol. **46**:930–940.
7. **Barber, M. A.** 1908. The rate of multiplication of *Bacillus coli* at different temperatures. J. Infect. Dis. **5**:379–400.
8. **Barer, R., K. F. A. Ross, and S. Tkaczyk.** 1953. Refractory of living cells. Nature (London) **171**:720–724.
9. **Bisset, K. A.** 1952. Bacteria. E & S Livingstone Ltd., Edinburgh, Scotland.
10. **Bowden, W. B.** 1977. Comparison of two direct-count techniques for enumerating aquatic bacteria. Appl. Environ. Microbiol. **33**:1229–1232.
11. **Boylen, C. W., and J. C. Ensign.** 1970. Long-term starvation of rod and spherical stage cells of *Arthrobacter crystallopoietes*. J. Bacteriol. **103**:569–577.
12. **Boylen, C. W., and J. C. Ensign.** 1970. Intracellular substrates for endogenous metabolism during long-term starvation of rod and spherical cells of *Arthrobacter crystallopoieties*. J. Bacteriol. **103**:578–587.
13. **Boylen, C. W., and M. H. Mulks.** 1978. The survival of coryneform bacteria during periods of prolonged nutrient starvation. J. Gen. Microbiol. **105**:323–334.
14. **Bretz, H. W.** 1962. Simple method for estimating slide culture survival. J. Bacteriol. **84**:1115–1116.
15. **Brock, T. B., and M. L. Brock.** 1966. Autoradiography as a tool in microbial ecology. Nature (London) **209**:734–736.
16. **Buchanan, R. E.** 1918. Life phases in a bacterial culture. J. Infect. Dis. **23**:109–125.
17. **Buck, J. D.** 1979. The plate count in aquatic microbiology, p. 19–28. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity, and ecology. American Society for Testing Materials, Philadelphia.
18. **Butkevich, N. V., and V. S. Butkevich.** 1936. Multiplication of sea bacteria depending on the composition of the medium and on temperature. Microbiology (Moscow) **5**:322–343.
19. **Butkevich, V. S.** 1938. On the bacterial populations of the Caspian and Azov Seas. Microbiology (Moscow) **7**:1005–1021.
20. **Cabelli, V. J., A. P. Dufour, L. J. McCabe, and M. A. Levin.** 1982. Swimming-associated gastroenteritis and water quality. Am. J. Epidemiol. **115**:606–616.
21. **Calcott, P. H., and J. R. Postgate.** 1972. On substrate-accelerated death in *Klebsiella aerogenes*. J. Gen. Microbiol. **70**:115–122.
22. **Carlucci, A. F.** 1974. Nutrients and microbial response to nutrients in seawater, p. 245–248. *In* R. R. Colwell and R. Y. Morita (ed.), Effect of the ocean environment on microbial activities. University Park Press, Baltimore.
23. **Carlucci, A. F., and P. M. Williams.** 1978. Simulated *in situ* growth rates of pelagic marine bacteria. Naturwissenschaften **65**:541.
24. **Casida, L. E.** 1971. Microorganisms in unamended soil as observed by various forms of microscopy and staining. Appl. Microbiol. **21**:1040–1045.
25. **Casida, L. E.** 1965. Abundant microorganisms in soil. Appl. Microbiol. **13**:327–334.
26. **Chapman, A. G., L. Fall, and D. E. Atkinson.** 1971. Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bacteriol. **108**:1072–1086.
27. **Chrzanowski, T. H., R. D. Crotty, J. G. Hubbard, and R. P. Welch.** 1984. Applicability of the fluorescein diacetate method of detecting active bacteria in freshwater. Microb. Ecol. **10**:179–185.
28. **Cohen, S. S., and H. D. Barner.** 1954. Studies on unbalanced growth in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **40**:179–185.
29. **Collins, V. G., and C. Kipling.** 1957. The enumeration of water borne bacteria by a new direct count method. J. Appl. Bacteriol. **20**:257–264.
30. **Colwell, R. R.** 1979. Human pathogens in the aquatic environment, p. 337–344. *In* R. R. Colwell and J. Foster (ed.) Aquatic microbial ecology. University of Maryland Sea Grant, College Park.
31. **Conn, H. J.** 1948. The most abundant groups of bacteria in soil. Bacteriol. Rev. **12**:257–273.
32. **Daley, R. J., and J. E. Hobbie.** 1975. Direct counts of aquatic bacteria by a modified epifluorescence technique. Limnol. Oceanogr. **20**:875–882.
33. **Dawes, E. A.** 1976. Endogenous metabolism and the survival of starved prokaryotes. Symp. Soc. Gen. Microbiol. **26**:19–53.
34. **Dawes, E. A., and D. W. Ribbons.** 1965. Studies on the endogenous metabolism of *Escherichia coli*. Biochem. J. **95**:332–343.
35. **Dawes, E. A.,and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. **10**:135–266.
36. **Dawson, M. P., B. A. Humphrey, and K. C. Marshall.** 1981. Adhesion: a tactic in the survival strategy of a marine vibrio during starvation. Curr. Microbiol. **6**:195–199.
37. **Dean, A. C. R., and C. N. Hinshelwood.** 1966. Growth, function and regulation in bacterial cells. Clarendon Press, Oxford.
38. **Deitz, W. H., T. M. Cook, and W. A. Goss.** 1966. Mechanism of action of nalidixic acid on *Escherichia coli*. III. Conditions required for lethality. J. Bacteriol. **91**:768–773.
39. **Delpy, L. P., G. Beranger, and M. Kaweh.** 1956. Bacterial growth phases. Ann. Inst. Pasteur (Paris) **91**:112–115.
40. **Drlica, K.** 1984. Biology of bacterial DNA topoisomerases. Microbiol. Rev. **48**:273–289.
41. **Dutton, R. J., G. Bitton, and B. Koopman.** 1983. Malachite green-INT (MINT) method for determining active bacteria in sewage. Appl. Environ. Microbiol. **46**:1263–1267.
42. **Ensign, J. C.** 1970. Long term starvation of rod and spherical cells of *Arthrobacter crystallopoietes*. J. Bacteriol. **103**:569–577.
43. **Felter, R. A., R. R. Colwell, and G. B. Chapman.** 1969. Morphology and round body formation in *Vibrio marinus*. J. Bacteriol. **99**:326–335.
44. **Felter, R. A., S. F. Kennedy, R. R. Colwell, and G. B. Chapman.** 1970. Intracytoplasmic membrane structure in *Vibrio marinus*. J. Bacteriol. **102**:552–560.
45. **Fletcher, M., and K. Marshall.** 1982. Are solid surfaces of ecological significance to aquatic bacteria? Adv. Microb. Ecol. **6**:199–236.
46. **Fliermans, C. B., and E. L. Schmidt.** 1975. Autoradiography and immunofluorescence combined for autecological study of single cell activity with *Nitrobacter* as a model system. Appl. Microbiol. **30**:676–684.
47. **Flowers, R. S., and Z. J. Ordal.** 1979. Current methods to detect stressed staphylococci. J. Food Prot. **42**:362–367.
48. **Foerster, H. F.** 1981. L-Arginine inhibition of the initiation of germination in species of *Thermoactinomyces thalophilus*, p. 289–293. *In* H. S. Levinson, A. L. Sonensheim, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
49. **Foot, D. H., and C. B. Taylor.** 1949. The influence of the composition of the medium on the growth of bacteria from water. Proc. Soc. Appl. Bacteriol. **1**:11–17.
50. **Francisco, D. E., R. A. Mah, and A. C. Rabin.** 1973. Acridine orange epifluorescent technique for counting bacteria in natural waters. Trans. Am. Microsc. Soc. **92**:416–421.
51. **Fraser, C. G.** 1920. The action of methylene-blue and certain other dyes on living and dead yeast. J. Physiol. Chem. **24**: 741–748.
52. **Freese, E., and Y. Frujita.** 1976. Control of enzyme synthesis during growth and sporulation, p. 164–184. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
53. **Fry, J. C., and T. Zia.** 1982. A method for estimating viability of aquatic bacteria by slide culture. J. Appl. Bacteriol. **53**: 189–198.
54. **Goss, W. A., W. H. Deitz, and T. M. Cook.** 1964. Mechanism of action of nalidixic action on *Escherichia coli*. J. Bacteriol. **88**:1112–1118.
55. **Goss, W. A., W. H. Deitz, and T. M. Cook.** 1965. Mechanism

of action of nalidixic acid on *Escherichia coli*. II. Inhibition of DNA synthesis J. Bacteriol. **89**:1068–1074.
56. **Gould, G. W.** 1969. Germination, p. 397–444. *In* G. W. Gould and A. Hurst (ed.) The bacterial spore. Academic Press, Inc., London.
57. **Gray, T. R. G.** 1976. Survival of vegetative microbes in soil. Symp. Soc. Gen. Microbiol. **26**:327–364.
58. **Griffiths, R. P., and R. D. Haight.** 1973. Reversible heat-injury in the marine psychrophilic bacterium, *Vibrio marinus* MP1. Can. J. Microbiol. **19**:557–561.
59. **Grimes, D. J., F. L. Singleton, and R. R. Colwell.** 1984. Allogenic succession of marine bacterial communities in response to pharmaceutical waste. J. Appl. Bacteriol. **57**:247–261.
60. **Guelin, A. M., I. E. Mishustina, L. V. Andreev, M. A. Bobyk, and V. A. Lambina.** 1979. Some problems of the ecology and taxonomy of marine microvibrios. Biol. Bull. Acad. Sci. USSR **5**:336–340.
61. **Hallock, F. A.** 1960. The life cycle of *Vibrio alternans* (sp. nov.). Trans. Am. Microsc. Soc. **79**:404–412.
62. **Harder, W., and L. Dukhizen.** 1983. Physiological responses to nutrient limitation. Annu. Rev. Microbiol. **37**:1–23.
63. **Harris, N. K., and E. O. Powell.** 1952. A culture chamber for the microscopical study of living bacteria with some observations on the spore-bearing aerobes. J. R. Microsc. Soc. **71**:407–420.
64. **Harrison. A. P., and F. R. Lawrence.** 1963. Phenotypic, genotypic, and chemical changes in starving populations of *Aerobacter aerogenes*. J. Bacteriol. **85**:742–750.
65. **Heinmets. F., W. W. Taylor, and J. J. Lehman.** 1953. The use of metabolites in the restoration of the viability of heat and chemically inactivated *Escherichia coli*. J. Bacteriol. **67**:5–14.
66. **Henrici, A. T.** 1923. Differential counting of living and dead cells of bacteria. Proc. Soc. Exp. Biol. Med. **20**:293–295.
67. **Henrici, A. T.** 1928. Morphological variation and the growth of bacteria. Charles C Thomas, Publisher, Springfield, Ill.
68. **Herson, D. C., and R. H. Baker.** 1982. New approaches to measuring total bacterial populations in water supplies. J. Am. Water Works Assoc. **74**:537–539.
69. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
70. **Hoppe, H. G.** 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of microautoradiography. Mar. Biol. **36**:291–302.
71. **Hoppe, H. G.** 1978. Relations between active bacteria and heterotrophic potential in the sea. Neth. J. Sea Res. **12**:78–98.
72. **Hurst. A.** 1977. Bacterial injury: a review. Can. J. Microbiol. **23**:935–944.
73. **Jannasch, H. W.** 1958. Studies on planktonic bacteria by means of a direct membrane filter method. J. Gen. Microbiol. **18**:609–620.
74. **Jannasch, H. W.** 1967. Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol. Oceanogr. **12**:264–271.
75. **Jannasch, H. W.** 1969. Estimations of bacterial growth rates in natural waters. J. Bacteriol. **99**:156–160.
76. **Jannasch, H. W.** 1979. Microbial ecology of aquatic low nutrient habitats, p. 243–260. *In* M. Shilo (ed.), Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim.
77. **Jannasch, H. W., and G. E. Jones.** 1959. Bacterial populations in seawater by different methods of enumeration. Limnol. Oceanogr. **4**:128–139.
78. **Jarnagin, J. L., and D. W. Luchsinger.** 1980. The use of fluorescein diacetate and ethidium bromide as a stain for evaluating viability of mycobacteria. Stain Technol. **55**:253–258.
79. **Jebb, W. H. H., and H. H. Tomlinson.** 1960. a microculture technique for observing the early growth of Mycobacteria. J. Gen. Microbiol. **22**:93–101.
80. **Jennison, M. W.** 1937. Relations between plate counts and direct microscopic counts of *Escherichia coli* during logarithmic growth period. J. Bacteriol. **33**:461–469.
81. **Jones. J. G.** 1974. Some observations on direct counts of freshwater bacteria obtained with a fluorescence microscope. Limnol. Oceanogr. **19**:540–543.
82. **Jones, J. G., and B. M. Simon.** 1975. An investigation of errors in direct counts of aquatic bacteria by epifluorescence microscopy, with reference to a new method for dyeing membrane filters. J. Appl. Bacteriol. **39**:317–329.
83. **Jones, K. L., and M. E. Rhodes-Roberts.** 1980. Physiological properties of nitrogen-scavenging bacteria from the marine environment. J. Appl. Bacteriol. **49**:421–433.
84. **Jones, K. L., and M. E. Rhodes-Roberts.** 1981. The survival of marine bacteria under starvation conditions. J. Appl. Bacteriol. **50**:247–258.
85. **Jones, P. C. T., and J. E. Mollison.** 1948. A technique for the quantitative estimation of soil microorganisms. J. Gen. Microbiol. **2**:54–69.
86. **Kantor, G. J., and R. A. Deering.** 1968. Effect of nalidixic acid and hydroxyurea on division ability of *Escherichia coli* Fil^+ and Lon^- strains. J. Bacteriol. **95**:520–530.
87. **Kelly, C. D., and O. Rahn.** 1932. The growth rate of individual bacterial cells. J. Bacteriol. **23**:147–153.
88. **Kennedy, S. F., R. R. Colwell, and G. B. Chapman.** 1970. Ultrastructure of a psychrophilic marine vibrio. Can. J. Microbiol. **16**:1027–1032.
89. **Keynan, A.** 1978. Spore structure and its relations to resistance, dormancy, and germination, p. 43–53. *In* G. Chambliss and J. C. Vary (ed.) Spores VII. American Society for Microbiology, Washington, D.C.
90. **Kjeldgaard, N. O.** 1961. The kinetics of RNA and protein formation in *Salmonella typhimurium* during the transition between different states of balanced growth. Biochim. Biophys. Acta **49**:64–76.
91. **Kjelleberg, S., B. A. Humphrey, and K. C. Marshall.** 1982. Effect of interfaces on small, starved marine bacteria. Appl. Environ. Microbiol. **43**:1166–1172.
92. **Kjelleberg, S., B. A. Humphrey, and K. C. Marshall.** 1983. Initial phase of starvation and activity of bacteria at surfaces. Appl. Environ. Microbiol. **46**:978–984.
93. **Kjelleberg, S., K. C. Marshall, and M. Hermansson.** 1985. Oligotrophic and copiotrophic marine bacteria—observations related to attachment. FEMS Microbiol. Lett. **31**:89–96.
94. **Knaysi, G.** 1935. A microscopic method of distinguishing dead from living bacterial cells. J. Bacteriol. **30**:193–206.
95. **Koch, A. L.** 1959. Death of bacteria growing in culture. J. Bacteriol. **77**:623–651.
96. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25**:415–420.
97. **Kogure, K., U. Simidu, and N. Taga.** 1980. Distribution of viable marine bacteria in neritic seawater around Japan. Can. J. Microbiol. **26**:318–323.
98. **Kogut, M., J. W. Lightbrown, and P. Isaacson.** 1965. Effects of dihydrostreptomycin treatment on the growth of *Escherichia coli* after removal of extracellular antibiotic. J. Gen. Microbiol. **39**:165–183.
99. **Korgaonkar, K. S., and S. S. Ranade.** 1966. Evaluation of acridine orange fluorescence test in viability studies on *Escherichia coli*. Can. J. Microbiol. **12**:185–190.
100. **Kurath, G., and Y. Morita.** 1983. Starvation-survival physiological studies of a marine *Pseudomonas* sp. Appl. Environ. Microbiol. **45**:1206–1211.
101. **Lamanna, C., and M. F. Mallette.** 1965. Basic bacteriology: its biological and chemical background, 3rd ed. The Williams & Wilkins Co., Baltimore.
102. **Little, J. W., and D. W. Mount.** 1982. The SOS regulatory system of *Escherichia coli*. Cell **29**:11–22.
103. **MacDonell, M. T., and M. A. Hood.** 1982. Isolation and characterization of ultramicrobacteria from a Gulf Coast estuary. Appl. Environ. Microbiol. **43**:566–571.
104. **MacDonell, M. T., and M. A. Hood.** 1984. Ultramicrovibrios in Gulf Coast estuarine waters: isolation, characterization, and incidence, p. 551–561. *In* R. R. Colwell (ed.), Vibrios in the

environment. Wiley Interscience, New York.
105. **Mager, J., M. Kuczynski, G. Schatzberg, and Y. Avi-dor.** 1956. Turbidity changes in bacterial suspensions in relation to osmotic pressure. J. Gen. Microbiol. **14**:69–75.
106. **Maki, J. S., and C. C. Remsen.** 1981. Comparison of two direct count methods for determining metabolizing bacteria in freshwater. Appl. Microbiol. **41**:1132–1138.
107. **Mandelstam, J.** 1958. Turnover of protein in growing and non-growing populations of *Escherichia coli*. Biochem. J. **69**:110–119.
108. **Marshall, K. C.** 1976. Interfaces in microbial ecology. Harvard University Press, Cambridge.
109. **Marshall, K. C.** 1979. Growth at interfaces, p. 281–290. *In* M. Shilo (ed.), Strategies of microbial life in extreme environments. Verlag Chemie, Berlin.
110. **Martin, P., and R. A. MacLeod.** 1984. Observations on the distinction between oligotrophic and eutrophic marine bacteria. Appl. Environ. Microbiol. **47**:1017–1022.
111. **Matin, A.** 1979. Microbial regulatory mechanisms at low nutrient concentrations as studied in chemostat, p. 323–340. *In* M. Shilo (ed.), Strategies of microbial life in extreme environments. Verlag Chemie, Berlin.
112. **Meyer-Reil, L.** 1978. Autoradiography and epifluorescence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl. Environ. Microbiol. **36**:506–512.
113. **Mishustina, I. E., and T. G. Kameneva.** 1981. Bacterial cells of minimal size in the Barents Sea during the polar night. Mikrobiologiya **50**:360–363.
114. **Monod, J.** 1949. The growth of bacterial cultures. Annu. Rev. Microbiol. **3**:371–394.
115. **Montague, M. D., and E. A. Dawes.** 1974. The survival of *Peptococcus prevotii* in relation to the adenylate energy charge. J. Gen. Microbiol. **80**:291–299.
116. **Morita, R. Y.** 1975. Psychrophilic bacteria. Bacteriol. Rev. **39**:144–167.
117. **Morita, R. Y.** 1976. Survival of bacteria in cold and moderate hydrostatic pressure environments with special reference to psychrophilic and barophilic bacteria, p. 299–326. *In* T. R. G. Gray and J. R. Postgate (ed.), The survival of vegetative microbes. The Society for General Microbiology, Symposium 26. Cambridge University Press, Cambridge.
118. **Morita, R. Y.** 1982. Starvation-survival of heterotrophs in the marine environment. Adv. Microb. Ecol. **6**:171–198.
119. **Mossel, D. A. A., and P. Van Netton.** 1984. Harmful effects of selective media on stressed microorganisms, p. 329–371. *In* M. H. E. Andrews and A. D. Russell (ed.), The revival of injured microbes. Academic Press, Inc., London.
120. **Munro, A. L., and T. D. Brock.** 1968. Distinction between bacterial and algal utilization of soluble substances in the sea. J. Gen. Microbiol. **51**:35–42.
121. **Nelson, D. R., M. Cumsky, and D. R. Zusman.** 1981. Development-specific localization of Myxobacterial hemagglutination during fruiting body formation of *Myxococcus xanthus*, p. 276–280. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
122. **Nelson, L. M., and D. Parkinson.** 1978. Effects of starvation on survival of three bacterial isolates from an arctic soil. Can. J. Microbiol. **24**:1460–1467.
123. **Novitsky, J. A., and R. Y. Morita.** 1976. Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine vibrio. Appl. Environ. Microbiol. **32**:617–662.
124. **Novitsky, J. A., and R. Y. Morita.** 1977. Survival of a psychrotrophilic marine vibrio under long-term nutrient starvation. Appl. Environ. Microbiol. **33**:635–641.
125. **Novitsky, J. A., and R. Y. Morita.** 1978. Possible strategy for the survival of marine bacteria under starvation conditions. Mar. Biol. **48**:289–295.
126. **Olson, B. H.** 1978. Enhanced accuracy of coliform testing in sea water by modification of most probable number method. Appl. Environ. Microbiol. **36**:438–444.
127. **Paerl, H. W.** 1974. Bacterial uptake of dissolved organic matter in relation to detrital aggregation in marine and freshwater systems. Limnol. Oceanogr. **19**:966–972.
128. **Pearce, T. W., and E. O. Powell.** 1951. New techniques for the study of growing microorganisms. J. Gen. Microbiol. **5**:91–103.
129. **Peele, E. R., and R. R. Colwell.** 1981. Application of a direct microscopic method for enumeration of substrate-responsive marine bacteria. Can. J. Microbiol. **27**:1071–1075.
130. **Peroni, C., and O. Laverello.** 1975. Microbial activities as a function of water depth in the Ligurian Sea: an autoradiographic study. Mar. Biol. **30**:37–50.
131. **Pirt, S. J.** 1965. The maintenance energy of bacteria in growing cultures. Proc. Soc. London **63**:224–231.
132. **Poindexter, J. S.** 1981. Oligotrophy: fast and famine existence. Adv. Microb. Ecol. **5**:63–89.
133. **Postgate, J. R.** 1967. Viability measurements and the survival of microbes under minimum stress. Adv. Microb. Physiol. **1**:1–24.
134. **Postgate, J. R.** 1969. Viable counts and viability, p. 611–628. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 1. Academic Press, Inc., London.
135. **Postgate, J. R.** 1976. Death in macrobes and microbes, p. 1–19. *In* T. R. G. Gray and J. R. Postgate (ed.), The survival of vegetative microbes. Cambridge University Press, Cambridge.
136. **Postgate, J. R., J. E. Crumpton, and J. R. Hunter.** 1961. The measurement of bacterial viabilities by slide culture. J. Gen. Microbiol. **24**:15–24.
137. **Postgate, J. R., and J. R. Hunter.** 1962. The survival of starved bacteria. J. Gen. Microbiol. **29**:233–263.
138. **Postgate, J. R., and J. R. Hunter.** 1964. Accelerated death of *Aerobacter aerogenes* starved in the presence of growth-limiting substrates. J. Gen. Microbiol. **34**:459–471.
139. **Powell, E. O.** 1956. A rapid method for determining the proportion of viable bacteria in a culture. J. Gen. Microbiol. **14**:153–160.
140. **Quesnell, L. B.** 1963. A genealogical study of clonal development of *Escherichia coli*. J. Appl. Bacteriol. **26**:127–151.
141. **Rahn, O., and M. N. Barnes.** 1933. An experimental comparison of different criteria of death in yeast. J. Gen. Physiol. **16**:579–592.
142. **Ramsey, A. J.** 1974. The use of autoradiography to determine the proportion of bacteria metabolizing in an aquatic habitat. J. Gen. Microbiol. **80**:363–373.
143. **Ray, B.** 1979. Methods to detect stressed microorganisms. J. Food Prot. **42**:346–355.
144. **Ray, B., J. J. Jeseski, and F. F. Busta.** 1971. Repair of injury in freeze-dried *Salmonella anatum*. Appl. Microbiol. **22**:401–407.
145. **Ray, B., and M. L. Speck.** 1972. Repair of injury induced by freezing *Escherichia coli* as influenced by recovery medium. Appl. Microbiol. **24**:258–263.
146. **Reasoner, D. J., and E. E. Geldreich.** 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. **49**:1–7.
147. **Reusch, R. N., C.-J. Su, and H. L. Sadoff.** 1981. Novel lipids of *Azotobacter vinelandii* cysts and their possible role, p. 281–284. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
148. **Ribbons, D. W., and E. A. Dawes.** 1963. Environmental and growth conditions affecting the endogenous metabolism of bacteria. Ann. N.Y. Acad. Sci. **102**:564–586.
149. **Roszak, D. B., D. J. Grimes, and R. R. Colwell.** 1984. Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. **30**:334–338.
150. **Sedar, A. W., and R. M. Burde.** 1965. The demonstration of the succinic dehydrogenase system in *Bacillus subtilis* using tetranitroblue tetrazolium combined with techniques of electron microscopy. J. Cell Biol. **27**:53–66.
151. **Setlow, P.** 1975. Energy and small-molecular metabolism during germination of *Bacillus* spores, p. 443–450. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
152. **Sieburth, J.** 1979. Sea microbes. Oxford University Press,

New York.
153. **Sieburth, J. M.** 1967. Seasonal selection of estuarine bacteria by water temperature. Exp. Mar. Biol. Ecol. **1**:98–121.
154. **Singleton, F. L., J. W. Deming, E. R. Peele, B. Cavari, B. Gunn, and R. R. Colwell.** 1983. Microbial communities in surface waters at the Puerto Rico dumpsite, p. 201–218. *In* I. W. Duedall, B. H. Ketchum, P. K. Park, and D. P. Keste (ed.), Wastes in the ocean, vol. 1. Industrial and sewage wastes in the ocean. John Wiley & Sons, Inc., New York.
155. **Skinner, F. A., P. C. T. Jones, and J. E. Mollison.** 1952. A comparison of a direct and a plate-counting technique for the quantitative estimation of soil microorganisms. J. Gen. Microbiol. **6**:261–271.
156. **Smucker, R. A., and S. L. Simon.** 1981. Ultrastructure of *Streptomyces bambergiensis* aerialspore envelope, p. 285–288. *In* H. S. Levinson, A. L. Sonensheim, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
157. **Spino, D. F.** 1985. Characterization of dysgonic, heterotrophic bacteria from drinking water. Appl. Environ. Microbiol. **50**:1213–1218.
158. **Stevenson, L. H.** 1978. A case for bacterial dormancy in aquatic systems. Microb. Ecol. **4**:127–133.
159. **Strugger, S.** 1948. Fluorescence microscope examination of bacteria in soil. Can. J. Res. **26**:188.
160. **Sussman, A. S., and H. O. Halvorson.** 1966. Spores: their domancy and germination. Harper & Row, Publishers, Inc., New York.
161. **Tabor, P. S., and R. A. Neihof.** 1982. Improved microautoradiographic method to determine individual microorganisms active in substrate uptake in natural waters. Appl. Environ. Microbiol. **44**:945–953.
162. **Tabor, P. S., and R. A. Neihof.** 1984. Direct determination of activities for microorganisms of Chesapeake Bay populations. Appl. Environ. Microbiol. **48**:1012–1019.
163. **Tabor, P. S., K. Ohwada, and R. R. Colwell.** 1981. Filterable marine bacteria found in the deep sea: distribution, taxonomy and response to starvation. Microb. Ecol. **7**:67–83.
164. **Taubeneck, U.** 1959. New grid replica for precise localization in slide culture. J. Bacteriol. **77**:506–508.
165. **Tempest, D. W., and O. M. Neijssel.** 1978. Eco-physiological aspects of microbial growth in aerobic nutrient-limited environments. Adv. Microb. Ecol. **2**:105–153.
166. **Tempest, D. W., O. M. Neijssel, and W. Zevenboom.** 1983. Properties and performance of microorganisms in laboratory culture: their relevance to growth in natural ecosystems, p. 119–152. *In* J. H. Slater, R. Whittenbury, and J. W. T. Wimpenny (ed.), Microbes in their natural environments. Cambridge University Press, Cambridge.
167. **Thomas, T. C., and R. D. Batt.** 1969. Metabolism of exogenous arginine and glucose by starved *Streptococcus lactis* in relation to survival. J. Gen. Microbiol. **58**:371–380.
168. **Thornton, H. G., and P. H. H. Gray.** 1934. The numbers of bacterial cells in field soils, as estimated by the ratio method. Proc. Soc. Ser. B **115**:522–543.
169. **Torrella, F., and R. Y. Morita.** 1981. Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in sea water. Appl. Environ. Microbiol. **41**:518–527.
170. **Tuckett, J. D., and W. E. C. Moore.** 1959. Production of filterable particles by *Cellovibrio gilvus*. J. Bacteriol. **77**:227–229.
171. **United States Environmental Protection Agency.** 1976. Quality criteria for water. Government Printing Office, Washington, D.C.
172. **Vaccaro, R. F., and H. W. Jannasch.** 1966. Studies on heterotrophic activity in sea water based on glucose assimilation. Limnol. Oceanogr. **11**:596–607.
173. **Valentine, R. C., and J. R. G. Bradfield.** 1954. The urea method for bacterial viability counts with the electron microscope and its relation to other viability counting methods. J. Gen. Microbiol. **11**:349–357.
174. **Van Niel, C. B.** 1949. The kinetics of growth of microorganisms. *In* A. K. Parpart (ed.), The chemistry and physiology of growth. Princeton University Press, Princeton, N.J.
175. **Wade, H. E., and D. M. Morgan.** 1954. Differentiation of growing and non-growing bacteria by a staining method. Nature (London) **174**:920–921.
176. **Waksman, S. A.** 1927. Principles of soil microbiology. The Williams & Wilkins Co., Baltimore.
177. **Weibull, C.** 1953. Observations on the staining of *Bacillus megaterium* with triphenyltetrazolium. J. Bacteriol. **66**:137–139.
178. **White, P. B.** 1947. A method for combined positive and negative staining of bacteria. J. Pathol. Bacteriol. **59**:334–335.
179. **Williams, P. J. L., and R. W. Gray.** 1970. Heterotrophic utilization of dissolved organic compounds in the sea. II. Observations on response of the heterotrophic marine population to abrupt increase in amino acid concentration. J. Mar. Biol. Assoc. U.K. **50**:871–881.
180. **Winslow, C.-E. A., and O. R. Brooke.** 1927. The viability of various species of bacteria in aqueous suspension. J. Bacteriol. **13**:147–171.
181. **Winslow, C.-E. A., and H. H. Walker.** 1939. The earlier phases of the bacterial culture cycle. Bacteriol. Rev. **3**:147–171.
182. **Wood, E. J. F.** 1965. Marine microbiology: modern biological studies. Chapman and Hall, Ltd., London.
183. **Wright, R. T.** 1978. Measurements and significance of specific activity in the heterotrophic bacteria of natural waters. Appl. Environ. Microbiol. **36**:297–305.
184. **Xu, H.-S., N. C. Roberts, L. B. Adams, P. A. West, R. J. Siebeling, A. Huq, M. I. Huq, R. Rahman, and R. R. Colwell.** 1984. An indirect fluorescent antibody staining procedure for detection of *Vibrio cholerae* serovar O1 cells in aquatic environmental samples. J. Microbiol. Methods **2**:221–231.
185. **Xu, H.-S., N. Roberts, F. L. Singleton, R. W. Attwell, D. J. Grimes, and R. R. Colwell.** 1982. Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb. Ecol. **8**:313–323.
186. **Zimmerman, R., R. Iturriaga, and J. Becker-Birck.** 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36**:926–935.
187. **Zobell, C. E.** 1946. Marine microbiology. A monograph on hydrobacteriology. Chronica Botanica, Waltham, Mass.